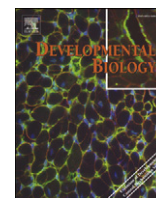


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## Developmental Biology

journal homepage: [www.elsevier.com/developmentalbiology](http://www.elsevier.com/developmentalbiology)Distinct roles for classical nuclear import receptors in the growth of multinucleated muscle cells<sup>☆</sup>Monica N. Hall<sup>a</sup>, Christine A. Griffin<sup>b</sup>, Adriana Simionescu<sup>b</sup>, Anita H. Corbett<sup>c,\*</sup>, Grace K. Pavlath<sup>d,\*\*</sup><sup>a</sup> Graduate Program in Genetics and Molecular Biology, Emory University, Atlanta, GA 30322, USA<sup>b</sup> Graduate Program in Biochemistry, Cell and Developmental Biology, Emory University, Atlanta, GA 30322, USA<sup>c</sup> Department of Biochemistry, Emory University, Atlanta, GA 30322, USA<sup>d</sup> Department of Pharmacology, Emory University, Atlanta, GA 30322, USA

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## ABSTRACT

Proper muscle function is dependent on spatial and temporal control of gene expression in myofibers. Myofibers are multinucleated cells that are formed, repaired and maintained by the process of myogenesis in which progenitor myoblasts proliferate, differentiate and fuse. Gene expression is dependent upon proteins that require facilitated nuclear import, however little is known about the regulation of nucleocytoplasmic transport during the formation of myofibers. We analyzed the role of karyopherin alpha (KPNA), a key classical nuclear import receptor, during myogenesis. We established that five karyopherin alpha paralogs are expressed by primary mouse myoblasts *in vitro* and that their steady-state levels increase in multinucleated myotubes, suggesting a global increase in demand for classical nuclear import during myogenesis. We used siRNA-mediated knockdown to identify paralog-specific roles for KPNA1 and KPNA2 during myogenesis. KPNA1 knockdown increased myoblast proliferation, whereas KPNA2 knockdown decreased proliferation. In contrast, no proliferation defect was observed with KPNA4 knockdown. Only knockdown of KPNA2 decreased myotube growth. These results identify distinct pathways involved in myoblast proliferation and myotube growth that rely on specific nuclear import receptors suggesting that regulation of classical nuclear import pathways likely plays a critical role in controlling gene expression in skeletal muscle.

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## Introduction

Skeletal muscle myofibers are multinucleated cells that contain many hundreds of nuclei spread across the length of the cell in a common cytoplasm. Each myonucleus is believed to express protein products for a defined volume of surrounding cytoplasm called the myonuclear domain (Allen et al., 1999). How a myofiber with hundreds of nuclei coordinates and regulates the transport of macromolecules into and out of these nuclei is unknown. Spatial and temporal regulation of nucleocytoplasmic transport into individual nuclei must occur within a single myofiber since transcriptional

activity of a specific gene locus can differ among nuclei within the same myofiber (Newlands et al., 1998). Furthermore, the transcription factors, NFATc1 and NFAT5, as well as the endonuclease EndoG localize to some myonuclei but not others within the same multinucleated cell (Abbott et al., 1998; Dupont-Versteegden et al., 2006; O'Connor et al., 2007). Skeletal muscle is a very plastic tissue that readily undergoes changes in mass in response to aging, injury or disease. Changes in muscle mass can impact breathing, locomotion and metabolism and affect motility and lifespan. Understanding how nucleocytoplasmic transport is regulated in myofibers will lead to a greater understanding of how external signals are sensed by muscle cells and translated into changes in gene expression necessary for tissue homeostasis.

All macromolecular transport between the nucleus and the cytoplasm is mediated by the nuclear pore complexes (NPC) that perforate the nuclear envelope (Lim and Fahrenkrog, 2006). Passive diffusion of ions and small molecules can occur; however, macromolecules require an appropriate targeting signal for transit through nuclear pores (Freitas and Cunha, 2009). Classical nuclear import, which is best characterized of the nuclear import mechanisms, depends on a classical nuclear localization signal (cNLS) in a cargo protein to be imported into the nucleus (Lange et al., 2007). cNLS motifs are comprised of basic residues present as either a single cluster (monopartite) or two clusters separated by a linker (bipartite)

**Abbreviations:** cNLS, classical nuclear localization signal; DM, differentiation media; eMyHC, embryonic myosin heavy chain; GM, growth media; KPNA, karyopherin alpha; KPNB1, karyopherin beta1; NFATc2, Nuclear Factor of Activated T cells cytoplasmic 2; NPC, nuclear pore complex.

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(Kalderon et al., 1984; Robbins et al., 1991). Proteins that contain a cNLS are imported into the nucleus by a heterodimeric receptor consisting of the classical nuclear import receptor karyopherin alpha (KPNA) and the pore targeting subunit karyopherin beta1 (KPNB1) (Fig. 1A) (Lange et al., 2007). Once in the nucleus, the cNLS-containing protein is released and both KPNA and KPNB1 are recycled separately back to the cytoplasm for another round of import (Hood and Silver, 1998; Kutay et al., 1997).

While *Saccharomyces cerevisiae* contains a single, essential karyopherin alpha, Srp1, the situation is more complex in *Homo sapiens* where a single KPNB1 can function with any of seven KPNA paralogs: KPNA1, KPNA2, KPNA3, KPNA4, KPNA5, KPNA6 and KPNA7 (Kelley et al., 2010; Kohler et al., 1997; Kohler et al., 1999; Tsuji et al., 1997). Six KPNA paralogs exist in mouse with which the corresponding human homologues share 80–90% amino acid identity (Fig. 1B) (Hu et al., 2010; Tsuji et al., 1997). KPNA paralogs in mouse and human are categorized into three subtypes based on their percentage of amino acid identity (Tsuji et al., 1997). Mouse subtypes are Subtype S: KPNA1 and KPNA6; Subtype P: KPNA2 and KPNA7; and Subtype Q: KPNA3 and KPNA4, with placement of recently discovered murine KPNA7 into its subtype being tentative (Hu et al., 2010). Subtype members share 80% to 90% amino acid identity, whereas different subtypes share 40% to 50% amino acid identity. While KPNA paralogs all function in classical nuclear import, their roles can differ in importing specific cNLS-containing proteins that are required for cell differentiation and function (Huenniger et al., 2010; Kohler et al.,

1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007).

To begin to understand how nucleocytoplasmic import is regulated in multinucleated muscle cells, we utilized an established *in vitro* model of myogenesis using primary mouse muscle cells (Rando and Blau, 1994). In this model, precursor mononucleated myoblasts proliferate in high serum-containing media but upon switching to a low mitogen media the cells exit the cell cycle, differentiate into myocytes that migrate and adhere to other myocytes and undergo membrane fusion to form multinucleated nascent myotubes. Further rounds of myocyte fusion with nascent myotubes yield large mature myotubes with many myonuclei. We used this model to analyze classical nuclear import in muscle cells, specifically the role of different KPNA subtypes represented by KPNA1, KPNA2 and KPNA4. This model offers the advantage that the role of KPNA-mediated nuclear import can be studied both in the context of mono- and multinucleated muscle cells. We determined that five mouse karyopherin alpha paralogs are expressed in primary myoblasts *in vitro* and their steady-state levels increase as myoblasts progress through myogenesis to form multinucleated myotubes. Through the use of RNAi, we demonstrate that KPNA1 and KPNA2 have differential roles in regulating myoblast proliferation as well as myotube size. Furthermore, we detect changes in the steady-state localization of a key cNLS-dependent cargo required for growth of myotubes, Nuclear Factor of Activated T cells, cytoplasmic 2 (NFATc2). In contrast to KPNA1 and KPNA2, knockdown of KPNA4 has no effect on myogenesis. These data provide evidence for distinct classical nuclear import pathways in skeletal muscle that rely on specific KPNA import receptors. We suggest that classical nuclear import may provide a novel regulatory mechanism during the formation and growth of multinucleated cells.

## Material and methods

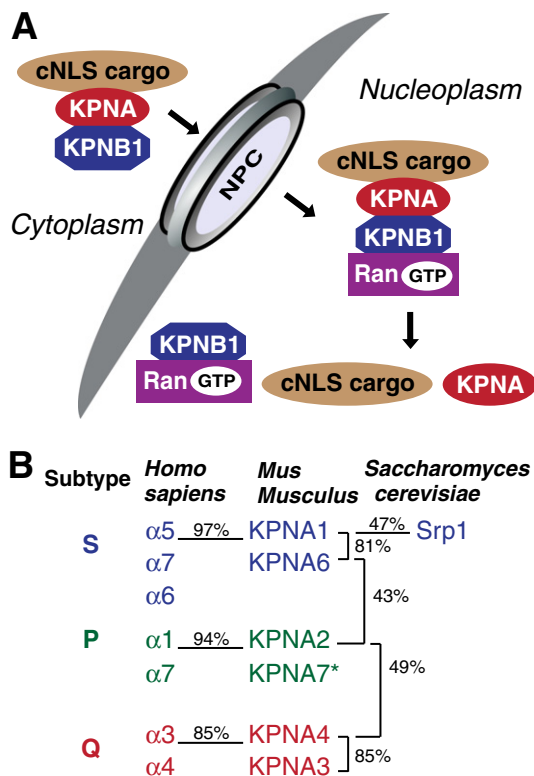
### Primary muscle cell culture

Primary myoblasts were isolated from the hind limb muscles of adult Balb/c mice between 8 and 12 wk of age as described previously (Jansen and Pavlath, 2006) and cultured in growth medium (GM: Ham's F-10, 20% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin and 100 µg/ml streptomycin) on collagen coated plates. Primary cultures were enriched for myogenic cells by using the preplating technique as described previously (Rando and Blau, 1994) and determined to be 97% pure by MyoD immunostaining.

To induce differentiation and fusion, myoblasts were seeded in GM onto 6-well plates ( $2 \times 10^5$  cells per well) coated with entactin–collagen–laminin (ECL, MilliPore) and allowed to adhere for ~1 h before switching to differentiation media [DM: DMEM, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% insulin–transferrin–selenium-A (Invitrogen)].

### RT-PCR analyses

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The reverse transcriptase reaction was performed using 2 µg of total RNA/sample using random hexamers and M-MLV reverse transcriptase (Invitrogen). cDNA was amplified using 2.5 µM of each primer and the Expand High Fidelity PCR system (Roche). All KPNA primers spanned intron–exon boundaries to control for genomic contamination and were specific to individual KPNA paralogs as determined by Blast search. In addition, RNA was tested by PCR for DNA contamination. Primer sequences were: KPNA1 (F: 5'-TCCTGCTTTCGGGCTGTGG-3' and R: 5'-GGGGTGGATGCTGCTGTCC-3'); KPNA2 (F: 5'-CTGCTGGGCCATTCCTACCTGA-3' and R: 5'-ACGCGGCTCTCTGTGCTT-3'); KPNA3 (F: 5'-CAAGGGCCGATGTG-GAGA-3' and R: 5'-CTGATGTGGGAATGGAGAGTGC-3'); KPNA4 (F:



**Fig. 1.** Classical nuclear import. (A) In the cytoplasm, proteins containing a classical nuclear localization sequence (cNLS) are recognized by the classical nuclear import receptor complex consisting of karyopherin alpha (KPNA) and karyopherin beta1 (KPNB1). KPNA recognizes and binds cNLS-containing proteins, while KPNB1 mediates nuclear import of the complex through interactions with the nuclear pore complex (NPC). In the nucleus, KPNB1 is bound by Ran-GTP which induces a conformational change that dissociates the import complex leading to release of the cNLS protein. (B) KPNA paralogs are categorized into three subtypes, S, P and Q. The percent identity between a few subtypes is shown for human, mouse and budding yeast to illustrate the homology between and within subtypes for KPNA paralogs. \* The placement of recently discovered mouse KPNA7 into subtype P is tentative (Hu et al., 2010).

5'-GGGCGGTGGGGAGACTC-3' and R: 5'-TGAGTAGCGGAACCAAGTGAG-GAA-3'); KPNA6 (F: 5'-GAGAACATTCCTCGGCTTGG-3' and R: 5'-CCGGAGGCAGACATTATAGC-3'); KPNA7 (F: 5'-TCCAGTACTTCTACT-GACCC-3' and R: 5'-GGTACTTATAGTTCTTCAGCCTCT-3'). Amplification cycles for KPNA1 and KPNA2 consisted of 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final termination step at 72 °C for 5 min. Amplification cycles for KPNA3 and KPNA6 consisted of 94 °C for 5 min, 26 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and a final termination step at 72 °C for 5 min. Amplification cycles for KPNA4 consisted of 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 56 °C for 60 s, 72 °C for 30 s and a final termination step at 72 °C for 5 min. Amplification cycles for KPNA7 consisted of 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s and a final termination step at 72 °C for 4 min. As an internal control, 18S cDNA was amplified with QuantumRNA 18S rRNA primers (Ambion). PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. Two independent experiments were analyzed each in duplicate.

### Immunoblotting

To isolate cytosolic and nuclear extracts, myoblasts were trypsinized and centrifuged at 500×g at 4 °C for 5 min. Cell pellets were washed twice with PBS, resuspended in Nonidet NP-40 lysis buffer (10 mM TrisCl, pH 7.4, 10 mM NaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 0.5% (v/v) NP-40), lightly vortexed and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 500×g at 4 °C for 5 min, followed by removal of the supernatant containing the cytoplasmic fraction. Nuclei were resuspended in Nonidet NP-40 lysis buffer by light vortexing, centrifuged at 500×g at 4 °C for 5 min and washed with PBS before being resuspended in RIPA-2 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl<sub>2</sub>, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) containing protease inhibitors (Mini complete; Roche). Total cell lysate collection and immunoblotting were performed as previously described (Jansen and Pavlath, 2006). After non-specific binding was blocked with blocking buffer [5% non-fat dry milk in Tris-buffered saline (TBS)] overnight at 4 °C, membranes were incubated in blocking buffer containing anti-KPNA1 (Novus Biologicals), anti-KPNA2 (MBL) or NF-κB p65 subunit (Wang et al., 2009) at 1:10,000 dilution or anti-KPNA4 (MBL) at 1:1000 dilution, for 1 h. Blots were washed in TBS containing 0.1% Tween 20 (TBS-T) and then incubated in either HRP-conjugated goat anti-mouse IgG (KPNA1), HRP-conjugated goat anti-rat IgG (KPNA2 and 4) or HRP-conjugated donkey anti-rabbit IgG (NF-κB p65) (Jackson ImmunoResearch) for 1 h. After washing with TBS-T, antibody binding was detected using enhanced chemiluminescence. To demonstrate equal protein loading and/or efficient separation of nuclear-cytoplasmic fractions, membranes were reprobed with antibodies against alpha-tubulin (Sigma), EF1-alpha (Upstate), GAPDH (Santa Cruz), or ZC3H14 (Leung et al., 2009). Three independent experiments were performed.

### Immunocytochemistry

Immunostaining for KPNA1, KPNA2, and NFATc2 was performed using TSA (Tyramide Signal Amplification) Red or Green (PerkinElmer). Myogenin immunostaining was performed using Vectastain ABC Kit (Vector Laboratories Inc.). Cells were fixed with either 3.7% formaldehyde for KPNA immunostaining or methanol for NFATc2 immunostaining for 10 min at room temperature and following successive washes with PBS, were incubated in blocking buffer (0.5% BSA, 0.1% Triton X-100 in PBS) containing either 5% donkey serum for KPNAs, 5% goat serum for NFATc2, or TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% blocking reagent) for myogenin for 1 h. The cells were incubated overnight at 4 °C in 10 µg/ml anti-KPNA1 (Novus), 1 µg/ml anti-NFATc2 (Thermo Scientific), 5 µg/ml anti-KPNA2 (MBL), 1:10 dilution anti-myogenin (F5D, Developmental Studies Hybridoma Bank) or appropriate isotype controls. Cells were

washed with PBS containing 0.2% Tween 20 (PBS-T) and incubated with appropriate secondary antibodies (Jackson ImmunoResearch) diluted 1/250 in PBS-T: KPNA1 (biotin-conjugated donkey anti-mouse IgG F(ab')<sub>2</sub> fragments), KPNA2 (biotin-conjugated anti-rat IgG F(ab')<sub>2</sub> fragments), myogenin (biotin-conjugated anti-mouse IgG F(ab')<sub>2</sub> fragments), NFATc2 (biotin-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragments). After washes with PBS-T, cells immunostained for KPNAs and NFATc2 were incubated in TNB for 1 h followed by incubation with HRP-conjugated streptavidin diluted 1:200 in TNB for 30 min followed by TSA Red or Green diluted 1:200 for 5 min. Cells immunostained for myogenin were incubated with Vectastain ABC solution (Vector Laboratories Inc.) after several PBS-T washes, followed by incubation with DAB Fast 3,3'-Diaminobenzidine (Sigma). Cells immunostained for KPNAs and NFATc2 were counterstained with 25 µM 4',6-diamidino-2-phenylindole (DAPI) in PBS-T to identify nuclei.

### Cell proliferation, differentiation and fusion assays

Primary myoblasts were seeded onto 6-well plates (1×10<sup>5</sup> cells/well) and transfected with 80 nM control scrambled siRNA or one of two siRNAs targeting the KPNA of interest (Stealth siRNA; Invitrogen) using Lipofectamine 2000 (Invitrogen), with up to three serial transfections performed 24–48 h apart. Myoblasts were cultured for 24–48 h after the last transfection to ensure optimal protein knockdown before performing experiments.

To analyze cell proliferation, transfected cells were seeded onto 6-well plates (2×10<sup>5</sup> cells/well), grown for 23 h and then labeled with 10 µM bromodeoxyuridine (BrdU) (Sigma) for 1 h. Subsequently, cultures were immunostained for BrdU as previously described (Mitchell and Pavlath, 2004) and the percentage of BrdU<sup>+</sup> cells was determined using fluorescence microscopy with >700 nuclei analyzed for each condition in three independent experiments.

To analyze differentiation and fusion, transfected cells were differentiated for 0, 24 and 48 h and immunostained for embryonic myosin heavy chain (eMyHC, F1.652, neat hybridoma supernatant, Developmental Studies Hybridoma Bank) as described previously (Horsley et al., 2001). The differentiation index was determined by counting the number of nuclei in eMyHC<sup>+</sup> cells as a percentage of the total nuclei analyzed. The fusion index was calculated by dividing the total number of nuclei in myotubes by the total number of nuclei analyzed. Over 300 nuclei were analyzed for each condition in three independent experiments.

### Cell migration and adhesion assays

Migration of myocytes was quantified using time-lapse microscopy as described (Griffin et al., 2009; Jansen and Pavlath, 2006). Primary myoblasts transfected with either control scrambled siRNA or KPNA2 siRNA were seeded onto 100 mm plates at low density and switched to DM for 24 h to generate myocytes in the absence of myotube formation. Images were acquired using an Axiovert 200 M microscope with a 0.30 NA 10X Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and camera (QImaging) with OpenLab 5.50 (Improvision) every 5 min for 3 h. Cell velocities were calculated in µm/h using ImageJ software by tracking the paths of approximately 20 mononucleated cells in each of three independent experiments.

Cell-cell adhesion was analyzed by incubating myocytes in suspension as described (Griffin et al., 2009). Duplicate aliquots of cells were taken at 20 minute intervals for 60 min and the numbers of single cells and cells in clusters were determined by phase-contrast microscopy for each condition in three independent experiments. Trypan blue staining was used at 0 and 60 min to determine cell viability and was not significant between control and siRNA conditions. All migration and adhesion assays were performed blinded.



### Image preparation and statistical analyses

Confocal images for KPNA immunocytochemistry were acquired using an Zeiss LSM510 META microscope with a 40× objective with LSM510 software (Carl Zeiss MicroImaging, Inc., Germany). Multi-channel z-stack fluorescent images through DAPI stained nuclei were summed using ImageJ software version 10.2 and globally processed for size, brightness and contrast using Photoshop 11.0 (Adobe).

Other images for KPNA immunocytochemistry were acquired using an Axioplan microscope with a 0.5 NA 20× Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and charge-coupled device camera (Carl Zeiss MicroImaging, Inc.) with Scion Image 1.63 (Scion Corp.). Images taken for proliferation, differentiation and fusion assays were acquired using an Axiovert 200 M microscope with a 0.30 NA 10× or 20× Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and camera (QImaging) with OpenLab 5.50 (Improvision). All images were globally processed for size, brightness and contrast using Photoshop CS3 (Adobe).

To determine statistical significance, data from multiple groups were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons using Sigma Stat (version 2.03). To determine significance for two groups, comparisons were made using unpaired Student's t-test. For all statistical tests,  $p < 0.05$  was accepted for significance.

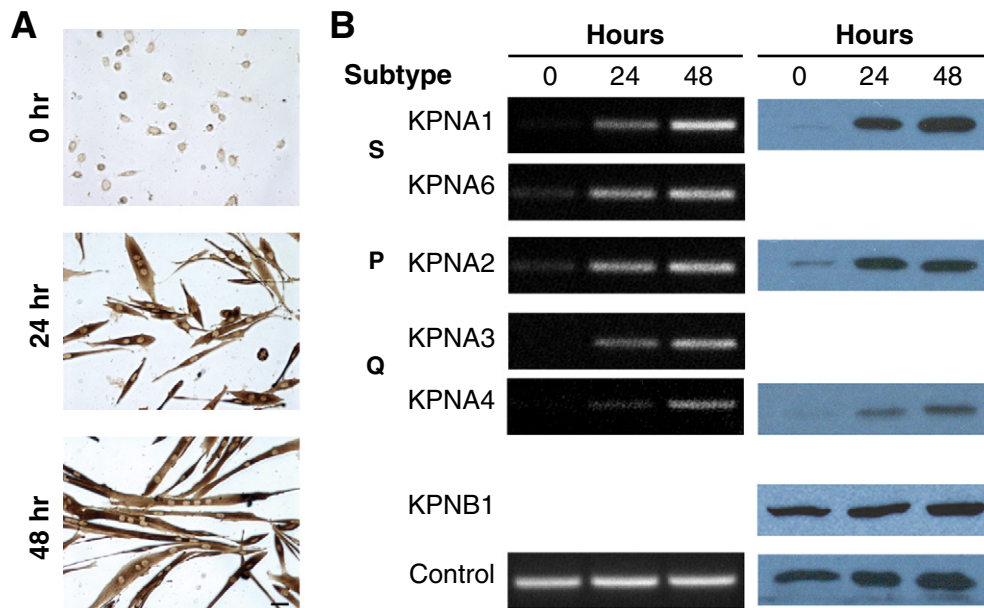
### Results

#### Five karyopherin alpha paralogs and KPNB1 are expressed during myogenesis

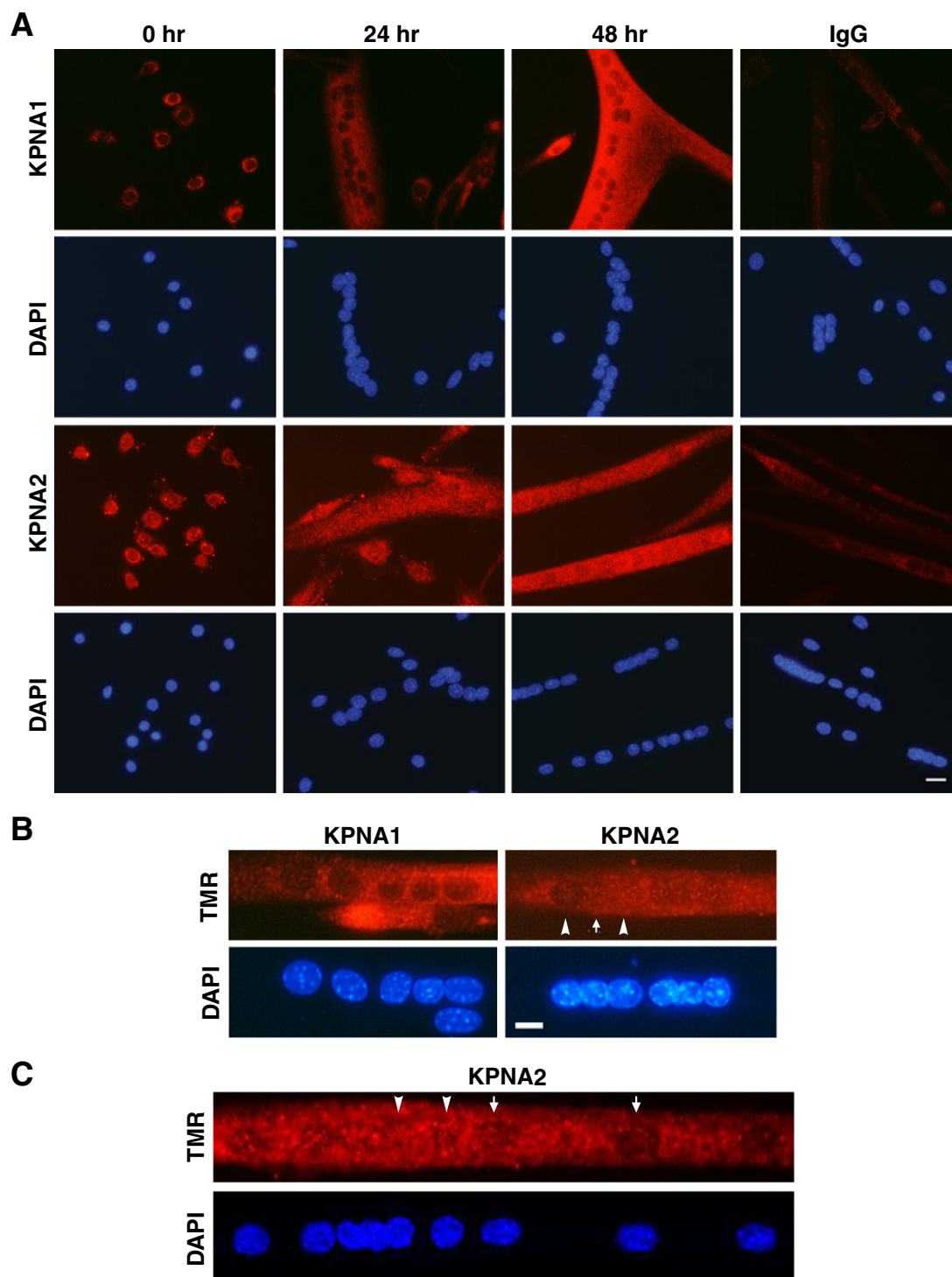
To determine whether the key transport receptors required for classical nuclear transport are expressed during myogenesis, we analyzed highly purified primary mouse muscle cells using RT-PCR and immunoblotting. RT-PCR was performed on total RNA isolated at 0, 24 and 48 h of differentiation using primers specific for each of the six karyopherin alpha paralogs. The 0-hour timepoint represents proliferating myoblasts, the 24-hour timepoint represents a mix of unfused myocytes and small multinucleated nascent myotubes, and

the 48-hour timepoint represents mainly large mature myotubes (Fig. 2A). mRNA for five KPNA paralogs was detected at all timepoints, with increased amounts detected in differentiated cultures (Fig. 2B), while mRNA for KPNA7, an ovary-specific paralog (Hu et al., 2010), was not detected at any timepoint (Supplemental Fig. S1). To analyze the steady-state levels of KPNA and KPNB1 proteins, immunoblotting was performed to detect KPNB1 and one representative KPNA paralog for each subtype: KPNA1 (Subtype S), KPNA2 (Subtype P) and KPNA4 (Subtype Q). These particular KPNA paralogs were chosen for analysis because these are the only ones for which monoclonal antibodies exist that have been verified to be paralog-specific (Kamikubo et al., 2004; Tachibana et al., 2005). Protein lysates were collected at 0, 24 and 48 h of differentiation and immunoblotting was performed. KPNA1, KPNA2 and KPNA4 were present at all timepoints with protein levels increasing with differentiation, consistent with the increase in steady-state levels observed for their respective mRNA transcripts (Fig. 2B). In contrast, the steady-state levels of KPNB1 protein remained unchanged during myogenesis (Fig. 2B). Thus, the key transport receptors required for classical nuclear import are expressed throughout myogenesis. The increased steady-state levels of KPNA paralogs during myogenesis suggest a global increase in the demand for nuclear protein import during myogenesis.

To complement immunoblotting analysis, we examined the expression of KPNA1 and KPNA2 at the single cell level using immunocytochemistry. KPNA1 and KPNA2 were detected in all cells at 0, 24 and 48 h during myogenesis (Fig. 3A). The fluorescence intensities of KPNA1 and KPNA2 increased during myogenesis consistent with the increases detected in the steady-state levels of protein and mRNA. Neither KPNA1 nor KPNA2 showed gross change in intracellular localization over the course of muscle differentiation (Fig. 3A). However, KPNA2 appeared to be more highly concentrated in nuclei compared to KPNA1 at all stages examined, suggesting their roles in muscle cells may differ. Interestingly, the fluorescence intensity of KPNA2 differed among individual myonuclei within a myotube, suggesting that KPNA2-dependent import could vary among myonuclei sharing a common cytoplasm (Figs. 3B, C). In contrast, no significant difference in the fluorescence intensity of KPNA1 was observed among nuclei within a single myotube (Figs. 3A, B).



**Fig. 2.** Components of the classical nuclear import system are expressed during myogenesis. (A) Primary mouse myoblasts were differentiated for 0, 24 and 48 h and immunostained for embryonic myosin heavy chain, eMyHC, a marker of differentiation (bar, 50 μm). (B) Total RNA or protein was isolated from primary myoblasts at 0, 24 and 48 h of differentiation. RT-PCR was performed using primers specific for each karyopherin alpha paralog and 18S rRNA as an internal cDNA control. Immunoblotting was performed with antibodies specific for each KPNA paralog or KPNB1 with GAPDH antibody as a loading control. The steady-state levels of karyopherin alpha paralogs increased during myogenesis, whereas KPNB1 levels did not change.



**Fig. 3.** KPNA1 and KPNA2 nuclear steady-state levels differ during myogenesis. (A) Representative fluorescent images of primary myoblasts differentiated for 0, 24 and 48 h and immunostained for either KPNA1 or KPNA2 and counterstained with DAPI (bar, 50  $\mu$ m). KPNA1 and KPNA2 were detected in all cells with greater nuclear fluorescence observed for KPNA2 at all stages of myogenesis. (B) Representative fluorescent images of myotubes at 48 h immunostained for either KPNA1 or KPNA2 (TMR, tetramethylrhodamine) and counterstained with DAPI (bar, 10  $\mu$ m). KPNA2 appeared to be more highly concentrated in some myonuclei (arrowhead) compared to other myonuclei (arrow), whereas no significant difference in the fluorescence intensity of KPNA1 was observed among nuclei. (C) Confocal z-stacked images of myotubes at 48 h immunostained for KPNA2 (TMR, tetramethylrhodamine) and counterstained with DAPI (bar, 10  $\mu$ m). Fluorescence was captured in successive confocal slices through DAPI stained nuclei to compare KPNA2 immunostaining among nuclei. Confocal microscopy confirmed that KPNA2 was increased in some myonuclei (arrowhead) compared to other myonuclei (arrow).

#### KPNA paralogs differentially alter the steady-state localization of NFATc2

To investigate the role of KPNA nuclear import receptors in nucleocytoplasmic import in muscle cells, we analyzed the steady-state localization of several cNLS proteins in myoblasts or myotubes upon depletion of individual KPNA paralogs. We chose proteins on the

basis of two or more of the following criteria: known or predicted cNLS, nuclear localization dependent on classical nuclear import, or known role in myogenesis. The nuclear localization of the following proteins was studied: the muscle-specific transcription factor myogenin (Buckingham et al., 2003), the general transcription factors NF- $\kappa$ B (Bakkar and Guttridge, 2010) and NFATc2 (Rao et al., 1997),

and the microtubule binding protein, myopodin (Weins et al., 2001). We predict that a cNLS protein that requires KPNA paralogs to access the nucleus would have an altered steady-state nuclear localization upon depletion of particular KPNA. Since a cNLS-containing protein may depend solely on a specific KPNA paralogs to access the nucleus or may exploit multiple paralogs with one paralog being preferred (Kohler et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007), we performed RNAi-mediated knockdown of a representative member of each KPNA subtype.

Myoblasts were transfected with either control siRNA or siRNA targeting the KPNA of interest and significant knockdown of each paralog was obtained (Fig. 4A). The double band detected for KPNA2 in myoblast lysates at this exposure has been observed in different

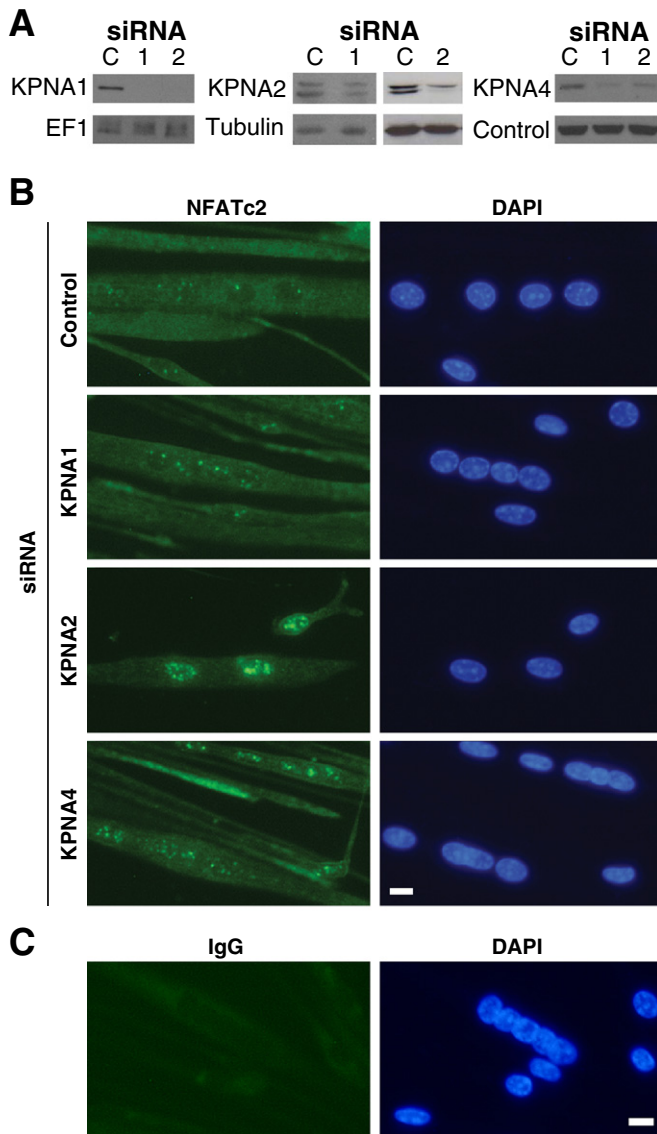
mouse and human tissues and is likely due to post-translational modification or alternative mRNA splicing (Kamei et al., 1999; Kohler et al., 1997). Off target effects against other KPNA paralogs during RNAi experiments were not detected as neither the steady-state level of protein nor mRNA of non-target KPNA was altered (Supplemental Fig. S2). Following transfection, cells were either analyzed as myoblasts or induced to differentiate for 24 or 48 h into myotubes depending on the particular cargo protein under investigation. The steady-state nuclear localization of candidate proteins was analyzed by immunocytochemistry of fixed cells or by immunoblotting of fractionated cells. No significant change in nuclear localization was observed for myogenin (Supplemental Fig. S3), NF- $\kappa$ B (Supplemental Fig. S3) or myopodin (data not shown). The lack of change in nuclear localization for these proteins with knockdown of a single KPNA may be due to redundancy among KPNA proteins for these particular cargoes as recently shown for the intracellular domain of Notch for which knockdown of three different KPNA was required to maximally decrease transcriptional activity (Huenniger et al., 2010). In contrast to our results with myogenin, NF- $\kappa$ B and myopodin, differences were noted with NFATc2, a protein whose cellular localization and role during myogenesis have been well characterized (Abbott et al., 1998; Horsley et al., 2001) and which is solely dependent on a characterized cNLS for nuclear import (Okamura et al., 2000; Torgerson et al., 1998). Following knockdown of KPNA1, KPNA2 or KPNA4, myotubes were immunostained with an antibody specific to NFATc2 (Tone et al., 2008) and counterstained with DAPI to locate nuclei. The fluorescence intensity of NFATc2 in nuclei was increased in both KPNA2 and KPNA4 siRNA-treated cultures compared to control cultures, with the most significant increase observed in cells depleted of KPNA2 (Figs. 4B, C). In contrast, the steady-state localization of NFATc2 in myotubes depleted of KPNA1 was similar to cells treated with control siRNA. Similar results for NFATc2 localization following KPNA knockdown were observed upon treatment of myotube cultures with ionomycin, a calcium ionophore that induces NFATc2 nuclear translocation (data not shown). Differences in NFATc2 steady-state nuclear localization in myotubes knocked down for KPNA1, KPNA2 or KPNA4 reveal specific roles for these KPNA paralogs in modulating the intracellular localization of NFATc2.

#### KPNA1 and KPNA2 regulate myoblast proliferation

The formation of multinucleated myotubes during myogenesis depends on an adequate pool of myoblasts which proliferate, differentiate and undergo cell–cell fusion. To investigate the role of KPNA paralogs in myoblast proliferation, we performed RNAi-mediated knockdown of KPNA1, KPNA2 or KPNA4. Cultures treated with each siRNA were labeled with bromodeoxyuridine (BrdU), a marker of S phase, and immunostained for BrdU to determine the percentage of cells actively replicating DNA (Fig. 5A). Knockdown of KPNA1 significantly increased the number of BrdU<sup>+</sup> cells by ~25% versus control siRNA, whereas KPNA2 siRNA-treated myoblasts displayed a ~25% decrease in proliferation. In contrast, no significant difference was observed in cells treated with KPNA4 (Fig. 5B). These data indicate that KPNA paralogs have non-redundant roles in regulating proliferation in primary muscle cells. Furthermore, KPNA1 and KPNA2 may have specific roles in the nucleocytoplasmic import of cNLS proteins required for the negative or positive regulation of proliferation, respectively.

#### KPNA2 regulates myotube size

To investigate the role of KPNA paralogs during myoblast differentiation and fusion to form multinucleated myotubes, we performed RNAi-mediated knockdown of KPNA1, KPNA2 or KPNA4 in myoblasts and then induced the cells to differentiate for 24 and 48 h. The 24-hour timepoint, representing a mix of unfused myocytes and



**Fig. 4.** Knockdown of KPNA paralogs alters the steady-state localization of NFATc2. (A) Primary myoblasts were transfected with either control scrambled siRNA or siRNAs directed against KPNA1, KPNA2 or KPNA4. Immunoblotting was performed with antibodies specific for each KPNA to determine the efficiency of knockdown. Antibodies targeting EF1- $\alpha$ ,  $\alpha$ -tubulin or a non-specific band (control) obtained during KPNA4 immunoblotting were used as a loading control. (B) Representative fluorescent images of primary myoblasts transfected with different siRNAs. Myoblasts were differentiated for 48 h and immunostained using antibodies targeting (B) NFATc2 or (C) IgG isotype control antibodies and counterstained with DAPI (bar, 25  $\mu$ m). NFATc2 was increased in the nuclei of cells treated with KPNA2 and KPNA4 siRNA compared to control with the greatest increase observed with KPNA2 siRNA; no differences were observed with KPNA1 siRNA.



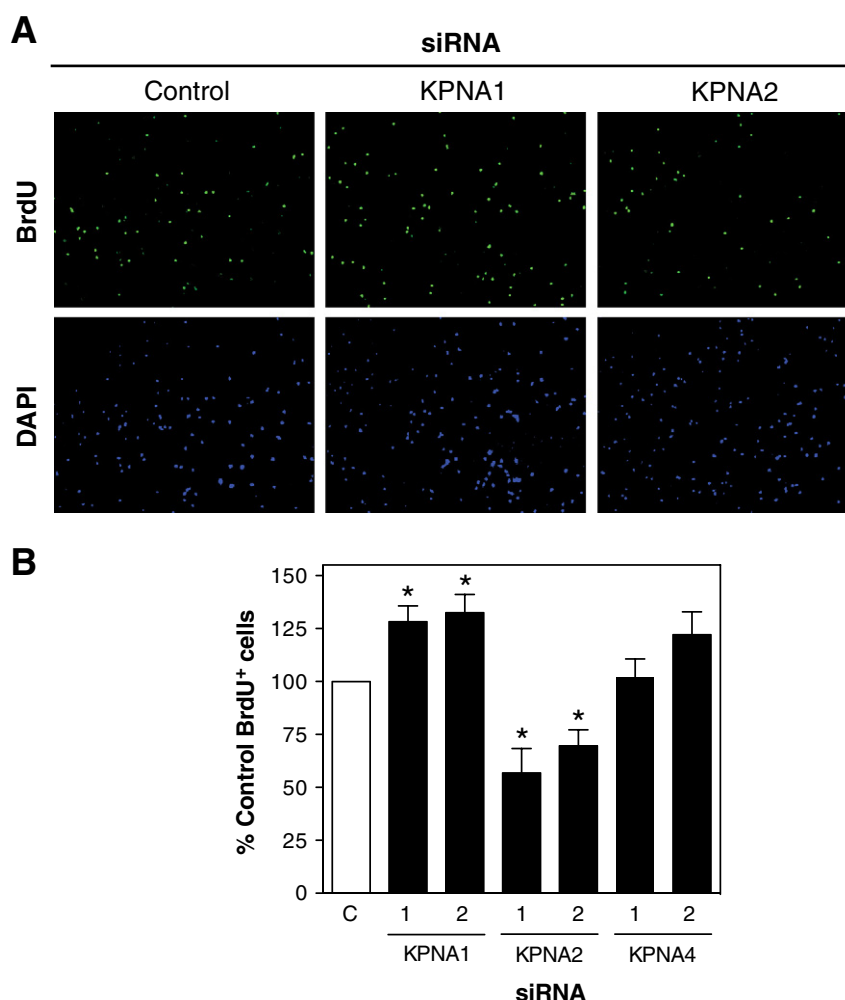
small multinucleated nascent myotubes, was used to assess early stages of differentiation and fusion, while the 48-hour timepoint representing mainly large mature myotubes was used to assess completion of myogenic fusion. Cultures treated with KPNA2 siRNA contained thinner myotubes than cultures treated with control siRNA at both 24 and 48 h (Fig. 6A), whereas no defects were observed in cultures treated with siRNAs directed against either KPNA1 or KPNA4 (data not shown). Since thin myotubes can correlate with differences in myonuclear number, cultures were immunostained for embryonic myosin heavy chain (eMyHC), which stains only the cytoplasm thereby facilitating the counting of nuclei to quantify the fusion index (Horsley et al., 2001). The fusion index indicates the percentage of cells that have fused to form multinucleated myotubes. While no significant difference in fusion was observed between KPNA1 or KPNA4 siRNA-treated cultures and control siRNA-treated cultures at either 24 or 48 h (Supplemental Fig. S4A), a significant decrease in fusion occurred in KPNA2 siRNA-treated cultures at both 24 and 48 h (Fig. 6B). These data indicate that KPNA2 has a distinct role in regulating myotube formation during myogenesis.

Myoblast proliferation and differentiation are upstream of myotube formation during myogenesis and defects in either could result in reduced myotube formation. However, the fusion defect observed for KPNA2 is not due to differences in cell number, since control siRNA and KPNA2 siRNA-treated cultures were plated at the same cell density and immediately induced to differentiate such that prolifer-

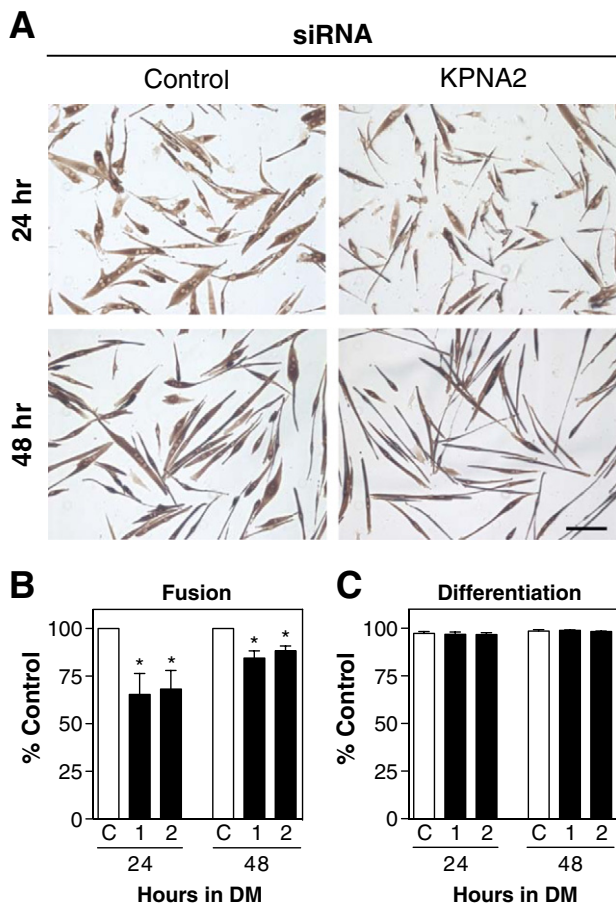
ation was not a factor. To investigate defects in myoblast differentiation, we determined the differentiation index, which indicates the percentage of cells that have differentiated as determined by immunostaining for the differentiation marker eMyHC (Horsley et al., 2001). We did not observe a defect in differentiation in KPNA2 siRNA-treated cultures at 24 or 48 h; therefore, the fusion defect observed upon KPNA2 knockdown is not due to a defect in differentiation (Fig. 6C). We also did not detect differentiation defects in KPNA1 or KPNA4 RNAi-treated cultures (Supplemental Fig. 4B). These data suggest these particular KPNA paralogs either do not import cargo proteins required for differentiation or they have redundant roles in this process. However, the fusion defect observed solely in KPNA2 siRNA-treated cultures reveals a specific role for KPNA2 in importing cNLS proteins involved in signaling pathways that regulate myotube formation and growth.

#### KPNA2 regulates myocyte migration

Migration and cell–cell adhesion are essential to myogenesis, where myoblasts exit the cell cycle, differentiate into myocytes that migrate, adhere to other myocytes and undergo membrane fusion to form multinucleated myotubes (Griffin et al., 2009; Jansen and Pavlath, 2006). Since altered cell adhesion could contribute to the reduction in myotube size upon depletion of KPNA2, we utilized a suspension based adhesion assay (Griffin et al., 2009), which allows



**Fig. 5.** KPNA paralogs have distinct roles in myoblast proliferation. (A) Primary myoblasts were transfected with either control scrambled siRNA or siRNAs (1 or 2) directed against KPNA1, KPNA2 or KPNA4. Cultures were labeled with BrdU and subsequently immunostained. Representative fluorescent images of BrdU immunostaining and nuclear counterstaining with DAPI are shown (bar, 100  $\mu$ m). (B) Knockdown of KPNA1 significantly increased the percentage of BrdU<sup>+</sup> cells while loss of KPNA2 significantly reduced the percentage of BrdU<sup>+</sup> cells. No significant difference was observed in cells treated with KPNA4 siRNA. Data are mean  $\pm$  SEM from three independent experiments, \* $p$  < 0.05.



**Fig. 6.** Knockdown of KPNA2 decreases myotube size. (A) Primary myoblasts were transfected with either control scrambled siRNA or one of two siRNAs (1 or 2) against KPNA2. Cells were differentiated for either 24 or 48 h in differentiation media (DM) and immunostained for eMyHC, (bar, 50  $\mu$ m). (B) The fusion index was significantly decreased with KPNA2 siRNAs at both 24 and 48 h of differentiation. (C) The differentiation index was not altered with KPNA2 siRNA. Data are mean  $\pm$  SEM from three independent experiments, \* $p < 0.05$ .

analysis of cell–cell adhesion without the confounding effects of cell migration. Myocytes treated with control siRNA or KPNA2 siRNA were suspended in media and aliquots were removed over a 60 minute timecourse to count the number of adhered or unadhered cells using phase contrast microscopy. No significant differences in adhesion, as measured by the percentage of single cells (Fig. 7A) or clusters (Fig. 7B), were noted between control siRNA and KPNA2 siRNA-treated cultures. Thus, KPNA2 does not appear to have a significant role in regulating cell–cell adhesion. Subsequently, we used time-lapse microscopy to examine whether the reduction in myotube size upon depletion of KPNA2 could be due to altered myocyte motility. Myocytes treated with KPNA2 siRNA migrated shorter distances than myocytes treated with control siRNA (Fig. 8A). In addition, KPNA2 siRNA-treated cultures contained more slowly migrating cells than those treated with control siRNA (Fig. 8B) with the mean velocity decreased by ~25% compared to control (Fig. 8C). Our data suggest that defects in myocyte migration may contribute to the fusion defect observed upon KPNA2 knockdown and that KPNA2 may import cNLS-containing proteins that regulate cell migration.

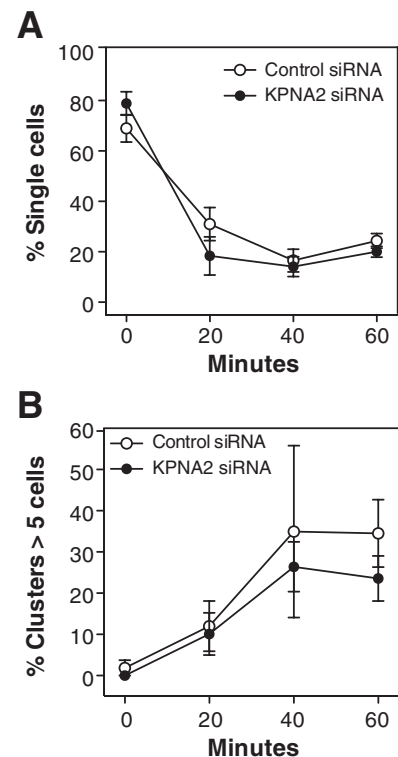
## Discussion

Control of gene expression during myogenesis is dependent upon nuclear proteins that require facilitated nuclear import. We determined that skeletal muscle utilizes the well characterized classical nuclear import pathway which is a prevalent mechanism for

facilitated nuclear protein import (Lange et al., 2007; Marfori et al., in press). Myoblasts and myotubes express the key transport receptors that are required for classical nuclear import, with non-redundant roles for specific KPNA in regulating distinct import pathways involved in myoblast proliferation and myotube growth.

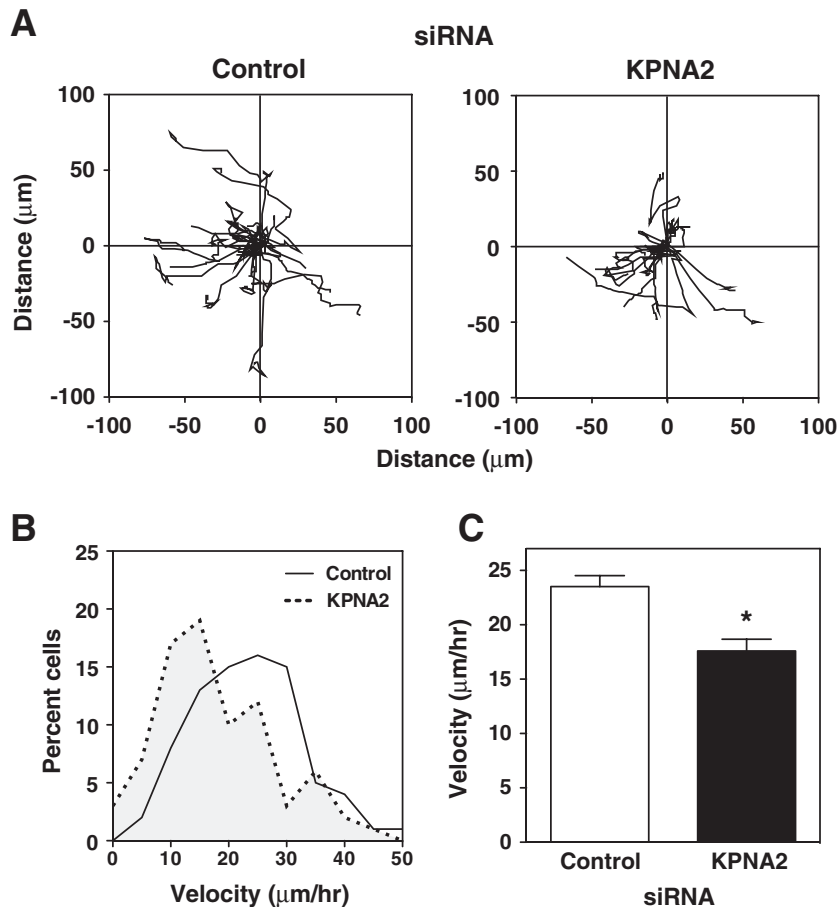
We show that five karyopherin alpha paralogs are expressed in primary mouse myoblasts and their steady-state levels increase in myotubes, suggesting a global increase in demand for classical nuclear import during myogenesis. These results differ from studies of differentiation in other cell types such as the cell line HL-60, keratinocytes, sperm and embryonic stem cells in which the steady-state levels of some KPNA increase with differentiation whereas others decrease (Hogarth et al., 2006; Kamei et al., 1999; Kohler et al., 1997; Kohler et al., 2002; Loveland et al., 2005; Okada et al., 2008; Suzuki et al., 2008; Umegaki et al., 2007; Yasuhara et al., 2007; Yasuhara et al., 2009). Such KPNA subtype switching during differentiation has been proposed to allow for differential nuclear import of transcription factors critical in either maintaining the undifferentiated state or inducing the differentiated state (Yasuhara et al., 2007). While no KPNA subtype switching occurs during muscle differentiation *in vitro*, as in monocyte cell differentiation (Nitahara-Kasahara et al., 2007), differential nuclear import of cNLS proteins required for various stages of myogenesis could still occur as individual KPNA paralogs may have different access to or affinities for cNLS proteins. Our results suggest that KPNA subtype switching is not a universal mechanism for modulating nuclear import of cargo proteins during cellular differentiation.

Our results and those of numerous studies support the idea that KPNA paralogs have non-redundant functions (Geles and Adam, 2001; Mason et al., 2002; Mason et al., 2003; Quensel et al., 2004; Ratan et al., 2008; Yasuhara et al., 2007). Three lines of evidence presented here suggest that karyopherin alphas have paralogue-specific



**Fig. 7.** Cell–cell adhesion is not altered following knockdown of KPNA2. (A) A suspension based assay was used to examine cell–cell adhesion over a 60-minute time course. No significant difference in either the percentage of single cells or (B) the percentage of cell clusters with >5 cells was observed with KPNA2 knockdown. Data are mean  $\pm$  SEM from three independent experiments.





**Fig. 8.** KPNA2 knockdown decreases muscle cell migration. (A) The migration of myocytes transfected with either control scrambled siRNA or siRNA against KPNA2 was analyzed using time-lapse microscopy. Twenty individual cell traces are shown for each siRNA condition. Myocytes treated with KPNA2 siRNA migrated shorter distances than control myocytes. (B) Histogram of the distribution of velocities for control or KPNA2 siRNA treated myocytes. Cells depleted of KPNA2 migrated more slowly than control myocytes. Data are mean  $\pm$  SEM of 80 cells (20 cells from each of four independent isolates). (C) The mean velocity of migration was reduced in KPNA2 siRNA treated cells compared to control. Data are mean  $\pm$  SEM of 80 cells (20 cells from each of four independent isolates), \* $p < 0.05$ .

roles in muscle. First, differences were observed in the nuclear localization of NFATc2 upon knockdown of individual KPNA, suggesting that KPNA have distinct roles in regulating the steady-state localization of NFATc2. Altered nuclear localization was observed with knockdown of KPNA2 and KPNA4, but not KPNA1. The increase in NFATc2 nuclear steady-state localization observed upon depletion of KPNA2 or KPNA4 could be indirect, perhaps due to changes in the import of other cNLS proteins that have a role in regulating the nuclear import or export of NFATc2. Consistent with our results showing increased nuclear localization of NFATc2 upon knockdown of single KPNA, Notch transcriptional activity was increased with knockdown of a single KPNA in muscle cells, but not with triple KPNA knockdown (Huenniger et al., 2010). Together these results illustrate the complexities in how nucleocytoplasmic transport of individual proteins is regulated.

A second line of evidence for non-redundant roles of KPNA paralogs during myogenesis is highlighted by the fact that knockdown of KPNA1 led to enhanced proliferation of myoblasts, whereas KPNA2 knockdown decreased myoblast proliferation and no change was observed with KPNA4 knockdown. These data suggest specific roles for KPNA1 and KPNA2 in importing cNLS-containing proteins involved in distinct signaling pathways that regulate myoblast proliferation. In contrast to our results, a study in Hela cells revealed that five of the seven human KPNA paralogs are required for cell proliferation, with a 25–80% decrease noted in cell number depending on the specific KPNA paralog analyzed (Quensel et al., 2004). However, our data provide the first evidence that a KPNA paralog,

specifically KPNA1, functions in a signaling pathway that negatively regulates proliferation. Indeed, KPNA can import molecules that negatively regulate proliferation, such as retinoblastoma protein and p27<sup>Kip1</sup> in other cell types (Hu et al., 2005; Shin et al., 2005). Whether KPNA1 is required for negatively regulating proliferation in cell types other than muscle will require further investigation. A recent study examining a KPNA1 knockout mouse (termed importin- $\alpha 5$  by the authors) revealed normal development of brain and other tissues, however, KPNA1 may have a different role in proliferation in the brain or compensation by other KPNA may have occurred during development since KPNA3 was up-regulated in the brain of these KPNA1 null mice (Shmidt et al., 2007). Our results using muscle cells reveal that individual KPNA participate in distinct import pathways that positively or negatively regulate myoblast proliferation, a crucial step for generating adequate cell numbers for proper formation and growth of multinucleated myotubes.

Finally, further non-redundancy among KPNA1, KPNA2 and KPNA4 was also noted as KPNA2 displayed a distinct role in regulating myotube size, since small, thin myotubes with reduced nuclear number were observed only upon depletion of KPNA2. The small myotubes observed with reduced KPNA2 are somewhat similar to the small myotube phenotype observed upon loss of NFATc2 (Horsley et al., 2001). However, upon KPNA2 knockdown, we observed an increase in the steady-state nuclear localization of NFATc2, suggesting that the phenotype we observed upon KPNA2 depletion is not due to reduced nuclear NFATc2, but possibly to altered nuclear import of multiple cNLS proteins involved in regulating both myotube

formation and growth. While our results indicate that a KPNA2-dependent pathway does not play a significant role in cell–cell adhesion, KPNA2 may import cNLS cargo proteins that regulate expression of structural or signaling proteins with a role in myocyte migration, or possibly cell–cell fusion, during myogenesis. Indeed, the reduction in cell velocity observed upon KPNA2 knockdown could contribute to the small myotube phenotype since decreased cell velocity would hinder myotube formation (Bae et al., 2008; Jansen and Pavlath, 2006). Similar to our results, decreased migration was observed upon depletion of KPNA2 in a lung cancer cell line (Wang et al., 2010). Whether similar KPNA2-dependent signaling pathways function in other cell types that migrate or undergo cell–cell fusion will require further study.

An alternate explanation for the phenotypes we observe during knockdown of KPNA1 or KPNA2 is that depletion of specific paralogs simply decreases the total pool of cellular KPNA. While we cannot formally rule out this possibility, our finding that depletion of KPNA1 increases cell proliferation whereas the depletion of KPNA2 decreases cell proliferation argues against a general effect on the overall pool of cellular KPNA. If the effects observed were simply due to a decrease in the overall pool of functional KPNA, one would expect that depletion of any KPNA paralog would alter cell function in the same manner, but potentially to different extents. Our finding that depletion of either KPNA1 or KPNA2 results in opposing proliferation phenotypes is counter to effects being due to general loss of KPNA and is more consistent with at least some paralog-specific function in classical nuclear protein import.

Identifying the cNLS-proteins responsible for the phenotypes observed upon depletion of KPNA1 or KPNA2 during myogenesis presents a great challenge since phenotypes are likely combinatorial due to the altered nuclear import of many cNLS proteins involved in regulating a multitude of genes required for proper myogenesis. While some nuclear proteins required for myogenesis have a characterized functional cNLS, such as Notch (Huenniger et al., 2010), and NFATc2 (Okamura et al., 2000), the vast majority of cNLS proteins have not been analyzed for their dependency upon specific KPNA for nuclear import. In *Mus musculus*, ~35–55% of nuclear proteins are predicted to use classical nuclear import (Marfori et al., in press), however, the contribution of the “cNLS importome” to the total nuclear proteome in muscle or other mammalian cells is unknown. Cataloging the “cNLS importome” for skeletal muscle and other cell types will be critical to revealing the prevalence of classical nuclear import and defining the role of individual KPNA paralogs in regulating cell fate and function.

The nuclear pore complex (NPC) and nuclear envelope serve as barriers to control nucleocytoplasmic transport and gene expression (Hetzer and Wente, 2009; Lim and Fahrenkrog, 2006). Nuclear envelope transmembrane proteins and nuclear pore proteins termed nucleoporins (Nups) have complex roles in nuclear organization, NPC biogenesis and modulating transport into and out of the nucleus (Hetzer and Wente, 2009). While our study is the first to investigate the role of soluble import receptors in skeletal muscle, the requirement for specific nuclear envelope transmembrane proteins and nucleoporins in myogenesis *in vitro* has been examined (Asally et al., 2011; Datta et al., 2009; Huber et al., 2009; Liu et al., 2009). Several nuclear envelope transmembrane proteins termed NETs are required for myogenic differentiation (Datta et al., 2009; Huber et al., 2009; Liu et al., 2009). Other nuclear envelope proteins linked to muscle function include emerin which is mutated in the X-linked form of Emery–Dreifuss Muscular Dystrophy (Manilal et al., 1996). Furthermore, the nucleoporin Nup358 is required for proper NPC architecture and myotube formation (Asally et al., 2011). While these studies highlight the critical role of specific nuclear envelope membrane proteins and nucleoporins in skeletal muscle, they do not address the role of soluble transport receptors in the complex regulation that must occur in multinucleated muscle cells.

In summary, the key transport receptors KPNA and KPNB1 that are required for classical nuclear import are expressed during *in vitro* myogenesis. KPNA paralogs display non-redundant roles in regulating distinct pathways involved in myoblast proliferation and myotube formation. These studies suggest that KPNA1 and KPNA2 transport critical cargo proteins required for different stages of myogenesis and that classical nuclear import likely has a key role in controlling gene expression in skeletal muscle. Our studies identify a novel regulatory pathway in skeletal muscle and lay the groundwork for unraveling the further complexities relating to classical nuclear import that likely exist in myofibers in skeletal muscle tissue *in vivo*. Questions remain as to how classical nuclear import regulates and adapts to the dynamic changes a multinucleated myofiber undergoes *in vivo* in response to skeletal muscle adaptation, aging and disease. Also unknown is whether classical nuclear import to different nuclei within a multinucleated myofiber varies depending on nuclear location within a myofiber or on the physiologic state of the muscle. We observed an increase in fluorescence intensity of KPNA2 in certain nuclei within a myotube which suggests that KPNA2-dependent protein import may differ among individual nuclei within a common cytoplasm. Differential classical nuclear import to individual myonuclei may contribute to transcriptional differences among nuclei within a myofiber (Newlands et al., 1998), such as nuclei localized at the neuromuscular synapse (Mejat et al., 2003) or myotendinous junction (Dix and Eisenberg, 1990), as well as nuclei undergoing apoptosis during muscle atrophy (Dupont-Versteegden et al., 2006). Thus, classical nuclear import is likely to emerge as an important regulatory pathway in multiple aspects of skeletal muscle biology. Understanding how classical nuclear import regulates gene expression in myofibers may lead to further insights into the mechanisms contributing to muscle growth and maintenance throughout the lifespan of an individual.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.06.032.

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